AMENDMENTS TO THE SPECIFICATION

Please replace the paragraphs beginning on page 2, line 30 and ending on page 5, line 2 with the following amended paragraphs:

The present invention relates to methods and compositions for wound healing, and in particular, methods and compositions to promote and enhance wound healing. For example, in some embodiments, the present invention provides a method for treating a wound, comprising providing an MMP prodomain peptide, and a subject having at least one wound; and administering the peptide to the subject under conditions such that the healing of the wound is promoted. In some embodiments, the subject is a patient with diabetes. In other embodiments, the subject is a burn patient. In still further embodiments, the wound is a chronic wound. In some embodiments, the peptide is derived from MMP 1 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-PRCGVPDVAQF-NH_2 (SEQ ID NO:1)). In other embodiments, the peptide is derived from MMP 7 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2)). In still further embodiments, the peptide is derived from MMP 2 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-PRCGNPDVANY-NH_2 (SEQ ID NO:3)). In yet other embodiments, the peptide is derived from MMP 9 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-PRCGVPDLGRFQ-NH_2 (SEQ ID NO:4)).

The present invention further provides a kit for the treatment of wounds in a subject, comprising an MMP prodomain peptide; and instructions for using the peptide to treat wounds in the subject. In some embodiments, the subject is a patient with diabetes. In other embodiments, the subject is a burn patient. In still further embodiments, the wound is a chronic wound. In some embodiments, the peptide is derived from MMP 1 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-PRCGVPDVAQF-NH_2 (SEQ ID NO:1)). In other embodiments, the peptide is derived from MMP 7 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-PRCGVPDVAEY-NH_2) (SEQ ID NO:2). In still further embodiments, the peptide is derived from MMP 2 (*e.g.*, including, but not limited to, a peptide

with the amino acid sequence Ac-PRCGNPDVANY-NH_2 (SEQ ID NO:3)). In yet other embodiments, the peptide is derived from MMP 9 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-PRCGVPDLGRFQ-NH_2 (SEQ ID NO:4)).

DESCRIPTION OF THE FIGURES

Figure 1 shows induction of human microvascular cell invasion by the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2). X axis, log of the peptide concentration in ng/ml. The peptide concentrations used are as follows: 2, 6, 20, and 60 ng/ml. Y axis, percent of microvascular cells invaded. Means are shown with first standard deviations.

Figure 2 shows inhibition of Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2)peptide–induced microvascular cell invasion by blocking anti MMP 1 COMY4A2 monoclonal antibody, but not by total serum IgG. X axis, media constituents: PRC, 600 ng/ml Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2); COMY4A2, 300 μg/ml anti MMP 1 monoclonal antibody; IgG, 300 μg/ml IgG. Y axis, percentage of invaded microvascular cells relative to the percentage invaded in the presence of 600 ng/ml Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2) peptide without blocking antibody. Means and first standard deviations are shown.

Figure 3 shows induction of human neonatal fibroblast invasion by the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2). X axis, log of the peptide concentration in ng/ml. The peptide used is the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2). The peptide concentrations used are as follows: 2, 6, 20, and 60 ng/ml. Y axis, percent of fibroblasts invaded. Means are shown with first standard deviations.

Figure 4 shows inhibition of Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2) peptide–induced fibroblast invasion by blocking anti MMP 1 COMY4A2 monoclonal antibody, but not by total serum IgG. X axis, media constitutents: PRC, 600 ng/ml Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2); COMY4A2, 300 μg/ml anti MMP 1 monoclonal antibody; IgG, 300 μg/ml IgG. Y axis, percentage of invaded fibroblasts relative to the percentage invaded in the presence of 600 ng/ml Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2) peptide without blocking antibody. Means and first standard deviations are shown.

Figure 5 shows induction of human keratinocyte invasion by the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2). X axis, log of the peptide concentration in ng/ml. The peptide used is the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2). The

peptide concentrations used are as follows: 2, 6, 20, and 60 ng/ml. Y axis, percent of keratinocytes invaded. Means are shown with first standard deviations.

Figure 6 shows inhibition of Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2) peptide–induced keratinocyte invasion by blocking anti MMP 1 COMY4A2 monoclonal antibody, but not by total serum IgG. X axis, media constitutents: PRC, 600 ng/ml Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2); COMY4A2, 300 μg/ml anti MMP 1 monoclonal antibody; IgG, 300 μg/ml IgG. Y axis, percentage of invaded keratinocytes relative to the percentage invaded in the presence of 600 ng/ml Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2) peptide without blocking antibody. Means and first standard deviations are shown.

Please replace the paragraph beginning on page 5, line 24 and ending on page 6, line 16 with the following amended paragraph:

The C57BL6Ks *db/db* mouse has been used extensively as a model of diabetic wounds in humans (Coleman, (1982) Diabetes 31: 1-6.). This mouse strain has a leptin receptor deficiency caused by a specific point mutation that affects splicing (Lee *et al.*, (1996) Nature 379: 632-635). *db/db* mouse wounds have been shown to have a number of molecular features in common with human diabetic wounds. For example, the wounds of *db/db* mice exhibit a decreased influx of inflammatory cells, as well as reduced growth factor expression (Fahey *et al.*, (1991) J. Surg. Res. 50: 308-313; Werner *et al.*, (1994) J. Invest. Dermatol. 103: 469-473). The demonstration that impaired healing in *db/db* mice could be reversed by the topical application of polypeptide growth factors, including fibroblast growth factor–2 (FGF-2) and platelet–derived growth factor–BB (PDGF-BB) (Tsuboi *et al.*, (1990) J. Exp. Med. 172: 245-251), lead to the successful trial of platelet derived growth factor–BB in human diabetics (Smiell *et al.*, (1999) Wound Repair Regen. 7: 335-346). Also, the PHSRN (SEQ ID NO:5) sequence of the fibronectin cell–binding domain has been shown to induce α5β1 integrin fibronectin receptor–mediated fibroblast and keratinocyte invasion of the extracellular matrix *in vitro*, as well as stimulate the rapid reepithelialization and contraction of dermal wounds in *db/db* mice, when applied as a peptide, Ac-PHSRN-NH_2 (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545). The successful completion of reepithelialization in this impaired wound healing model has been interpreted as indicating avoidance of the destructive matrix metalloproteinase expression profile that limits closure and can result in chronic, non–healing wounds (Neely *et al.*, (2000) J. Burn Care &

Rehab. 21: 395-402). Thus, many investigators have found the C57BL6Ks*db/db* mouse strain to be a very useful model for evaluating the effects of topically added polypeptides on healing–impaired wounds, and predicting their effects in the wounds of human diabetics.

Please replace the paragraph beginning on page 6, line 28 and ending on page 7, line 12 with the following amended paragraph:

Many experimental findings indicate the importance of MMP–1 activity in reepithelialization. MMP–1 is expressed by keratinocytes migrating on collagen (Pilcher *et al.*, (1997) J. Cell Biol. 137: 1445-1457). Its accumulation has been shown to be stimulated by the interaction of the invasion–inducing, PHSRN (SEQ ID NO:5) sequence of the fibronectin cell–binding domain with the α5β1 integrin fibronectin receptor in normal epithelial cells and in their metastatic counterparts, as well as in fibroblasts (Livant *et al.*, (2000) Cancer Res. 60: 309-320; Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545). After secretion, MMP–1 has been shown to interact with the surface α2β1 integrin of keratinocytes during their migration on type I collagen (Dumin *et al.*, (2001) J. Biol. Chem. 276: 29368-29374). Also, MMP–1 has been shown to be substantially upregulated in motile keratinocytes at the leading edge of the new epithelium of wounds, and to be required for successful reepithelialization (Saarialho–Kere *et al.*, (1995) J. Invest. Dermatol. 104: 982-988; Agren *et al.*, Exp. Derm. 10: 337-348). Total MMP–1 activity has been shown to increase approximately 100–fold in the wounded skin of healthy human volunteers, relative to unwounded skin (Petri *et al.*, (1997) Exp. Dermatol. 6: 133-139).

Please replace the paragraph beginning on page 12, line 8 and ending on line 16 with the following amended paragraph:

In some embodiments, the present invention provides peptides derived from MMP proteins. The present invention is not limited to peptides derived from a particular MMP protein. Peptides and mimetics (See section II below) derived from a variety of MMP proteins including, but not limited to, MMP 1, MMP 2, MMP 7, and MMP 9 are contemplated. As described above, it is contemplated that peptides comprising cysteine switch sequences are particularly preferred for use in the compositions and methods of the present invention. Exemplary peptide sequences include, but are not limited to, Ac-PRCGVPDVAQF-NH_2 (SEQ ID NO:1) (MMP 1 prodomain

peptide), Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2) (MMP 7), Ac-PRCGNPDVANY-NH_2 (SEQ ID NO:3) (MMP2), and Ac-PRCGVPDLGRFQ-NH_2 (SEQ ID NO:4) (MMP 9).

Please replace the paragraph beginning on page 13, line 11 and ending on line 22 with the following amended paragraph:

The present invention also contemplates synthetic mimicking compounds that are multimeric compounds that repeat the relevant peptide sequence. In one embodiment of the present invention, it is contemplated that the relevant peptide sequence is PRCGV/NPDVA (SEQ ID NO:6) sequence of the MMP prodomain. As is known in the art, peptides can be synthesized by linking an amino group to a carboxyl group that has been activated by reaction with a coupling agent, such as dicyclohexylcarbodiimide (DCC). The attack of a free amino group on the activated carboxyl leads to the formation of a peptide bond and the release of dicyclohexylurea. It can be necessary to protect potentially reactive groups other than the amino and carboxyl groups intended to react. For example, the α-amino group of the component containing the activated carboxyl group can be blocked with a tertbutyloxycarbonyl group. This protecting group can be subsequently removed by exposing the peptide to dilute acid, which leaves peptide bonds intact.

Please replace the paragraph beginning on page 17, line 8 and ending on line 25 with the following amended paragraph:

The results are shown in Figures 1-6. Figure 1 shows induction of human microvascular cell invasion by the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2). The peptide used is the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2). The peptide concentrations used are as follows: 2, 6, 20, and 60 ng/ml. Figure 2 shows that Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2) peptide–induced microvascular cell invasion is inhibited by blocking anti MMP 1 COMY4A2 monoclonal antibody, but not by total serum IgG. Figure 3 shows that invasion of human neonatal fibroblasts is enhanced by the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2). Figure 4 shows that Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2) peptide–induced fibroblast invasion is inhibited by blocking anti MMP 1 COMY4A2 monoclonal antibody, but not by total serum IgG. Figure 5 shows the induction of human keratinocyte invasion by the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH_2 (SEQ ID

NO:2). The peptide used is the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2). The peptide concentrations used are as follows: 2, 6, 20, and 60 ng/ml. Figure 6 shows that Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2) peptide–induced keratinocyte invasion is inhibited by blocking anti MMP 1 COMY4A2 monoclonal antibody, but not by total serum IgG. In conclusion, the MMP 7 switch peptide induces dose-dependent invasion by human fibroblasts, keratinocytes, and microvascular cells. The inhibition was blocked by a MMP 1–specific monoclonal antibody, but not by serum IgG.

Please replace the paragraphs beginning on page 18, line 5 and ending on page 20, line 12 with the following amended paragraphs:

Each treatment group consisted of 10 mice with duplicate, dermal wounds of 12.5 to 16.5 mm^2 made in the shoulder area. Complete methods were as described (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545). Treated animal were treated with 10 μg switch (PRC) sequence from MMP2 (Ac-PRCGNPDVANY-NH_2) (SEQ ID NO:3). Wound areas were measured by integration of digital images.

B. Results

The results are shown in Figure 7. One treatment with the PRC peptide just after wounding decreased the time required for closure of all db/db wound by 50%, relative to untreated db/db mice. The closure of PRC-treated wounds appears to be as rapid as that of db/+ non-diabetic mice.

Example 3

Methods

This example described methods useful in performing some embodiments of the present invention.

Cell culture and SU-ECM invasion assays. In SU-ECM *in vitro* invasion substrates, a basement membrane surrounds a blastocoel, in which invading cells localize shortly after suspension and placement on the outer surfaces. Even in the presence of serum, these invasion substrates have been shown to be free of background invasion by normal cells (Livant *et al.*, (1995) *Cancer Res.* 55: 5085-5093; Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545; Livant *et al.*, (2000) Cancer

Res. 60: 309-320). These invasion substrates have been used to define the invasion–promoting activity of the PHSRN (SEQ ID NO:5) sequence from the fibronectin cell–binding domain, a previously unanticipated function of this sequence. Ac-PHSRN-NH_2 (SEQ ID NO:5) peptide has been shown to be a potent topical agent for stimulating dermal wound healing in obese diabetic C57BL6Ks*db/db* mice (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545). Because they are naturally serum–free, the SU-ECM invasion substrates have been used to define a potent inhibitor of plasma fibronectin–induced invasion by metastatic prostate cancer cells; and this inhibitor has been shown to be an effective and non–toxic anti-tumorigenic and anti-metastatic agent in Copenhagen rats (Livant *et al.* (2000) Cancer Res. 60: 309-320), as well as in nude mice bearing human prostate cancer tumors (Windsor *et al.*, (1991) Biochem. 30: 641-647; 38).

In vitro invasion assays are performed using SU-ECM with normal human fibroblasts, keratinocytes, and endothelial cells as previously described (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545). Undifferentiated, first or second passage human keratinocytes are obtained from the laboratory of C. Marcelo at the University of Michigan. Keratinocytes are cultured in serum-free keratinocyte growth medium (Clonetics, San Diego CA). Neonatal fibroblasts are obtained from the laboratory of J. Varani at the University of Michigan. First or second passage neonatal fibroblasts are cultured as previously described (Livant *et al.*,(1995) *Cancer Res.* 55: 5085-5093). Normal human endothelial cells (microvascular cells) are obtained from the laboratory of D. Arenberg at the University of Michigan. Microvascular cells are cultured as described (White *et al.*, (2001) J. Immunol. 16: 5362-5366). Cells from single cell suspensions (made with 0.25% trypsin/EDTA from Gibco Life Technologies, Grand Island NY) are rinsed, and briefly prebound to the following peptides (1, 3, 4, 5): Ac-PRCGVPDVAQF-NH_2 (SEQ ID NO:1) (from MMP–1), Ac-PRCGVPDVAEY-NH_2 (SEQ ID NO:2) (from MMP–7), Ac-PRCGNPDVANY-NH_2 (SEQ ID NO:3) (from MMP–2), Ac-PRCGVPDLGRFQ-NH_2 (SEQ ID NO:4) (from MMP–9). The testing of the peptides containing the C–to–S substitution is used to determine if invasion–promoting activities result from their interactions with the cysteine switch of inactive MMP–1. Thus, the effects on invasion of the corresponding 4 peptides containing the C–to–S substitution at the third position: Ac-PR<u>S</u>GVPDVAQF-NH_2 (SEQ ID NO:7), Ac-PR<u>S</u>GVPDVAEY-NH_2 (SEQ ID NO:8), Ac-PR<u>S</u>GNPDVANY-NH_2 (SEQ ID NO:9), and Ac-PR<u>S</u>GVPDLGRFQ-NH_2 (SEQ ID NO:10) is also assayed. To verify the lack of activity in the peptides containing the C–to–S substitution at the third position, peptides consisting of the

arbitrarily scrambled sequences from the MMP–1, –7, –2, and –9 switch region propeptides listed above are also tested. Final peptide concentrations in the SU-ECM invasion assays will range from 2 to 60 ng per ml. These concentrations are derived from the dose response curve shown in Figure 1 of Example 1. After prebinding to each of the peptides listed above, fibroblasts, endothelial cells, and keratinocytes are placed on SU-ECM invasion substrates in the appropriate media for 4 hours at 37°C, the time required to observe maximal invasion percentages (Livant *et al.*, (1995) *Cancer Res.* 55: 5085-5093). Cellular viability in SU-ECM invasion assays are next determined, and are expected to range from 90% to 99% (Livant *et al.*, (1995) *Cancer Res.* 55: 5085-5093). Invasion percentages are the ratio of the number of cells located in the blastocoelic cavities of SU-ECM invasion substrates to the total number of single cells adhering to them on their exterior and their interior surfaces. Each invasion percentage is the result of 3 to 4 independent determinations involving the scoring of the positions of all individual cells adhering to SU-ECM, typically about 100 individual cells for each determination.

Please replace the paragraph beginning on page 21, line 4 and ending on line 14 with the following amended paragraph:

Use of function–blocking antibodies in invasion assays The effects of function–blocking anti MMP 1 (COMY 4A2) antibody on Ac-PRCGVPDVAQF-NH_2 (SEQ ID NO:1) (MMP–1 switch peptide)–induced invasion is determined for keratinocytes, fibroblasts, and microvascular cells, placed on SU-ECM *in vitro* invasion substrates. To assess the specificity of the role of MMP–1 in switch peptide–induced invasion, the effects of function–blocking anti MMP 2 (CA 4001), and MMP 9 (GE 213) monoclonal antibodies (Schnaper *et al.*, (1993) J. Cell. Physiol. 156(2): 235-246) are also determined. All of the antibodies are purchased from Chemicon International (Temecula CA). Antibodies are prebound to cells for 30 minutes on ice in concentrations ranging from 10 to 300 μg per ml, prior to their placement on invasion substrates, according to established procedures (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545, 11, 25).

Please replace the paragraphs beginning on page 22, line 22 and ending on line page 23, line 28 with the following amended paragraphs:

Db/db mice are aged at least an additional 2 weeks prior to use in wound healing experiments, to make certain that they display the impaired wound healing phenotype characteristic of this strain. Treatment groups consist of 10 to 20 dermal wounds each, or 5 to 10 *db/db* or *db/+* mice with duplicate dermal wounds. Anesthetized mice are wounded once or twice on the upper back by pinching the skin away from the underlying fascia and muscle, and pushing the biopsy punch through the skin as it lies over a forefinger. When wounded without penetrating the fascia or underlying muscle, bleeding is not apparent in dermal wounds in *db/db* and *db/+* mice (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545). Thus, added peptide is not expected to be significantly diluted as it is delivered to wounds. As previously judged by measuring sections of day–old wounds with a reticle at 400–fold magnification (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545), *db/db* mice are expected to be wounded to a mean depth of approximately1.7 mm by this procedure. The depth of the wounds in the *db/+* mice is expected to be somewhat less because of the reduced amount of subcutaneous fat in these animals. However, in no case is underlying muscle wounded. In each experiment, the mice are age–matched. Based on preliminary dose response studies with the MMP–7 switch region propeptide, approximately 10 μg of the Ac-PRCGVPDVAQF-NH_2 (SEQ ID NO:1) peptide from MMP–1 proprotein switch region is added to wounds in a volume of 5 μ l normal saline (NS). The corresponding peptide containing the C–to–S substitution at the third position, Ac-PRSGVPDVAQF-NH_2 (SEQ ID NO:7) is also assessed for its ability to promote *db/db* wound healing. To verify the lack of activity in the peptide containing the C–to–S substitution at the third position, a peptide consisting of the scrambled sequence from the MMP–1 proprotein switch region, Ac-VVDAPPFCGQR-NH_2 (SEQ ID NO:11) is also tested. To assess the locality of the effect of switch region propeptide treatment on dermal wound healing, some *db/db* and *db/+* mice receive peptide in only 1 of 2 duplicate wounds; while the remaining wound on each mouse receive 5 μ l of NS only. This strategy has been previously used to demonstrate the locality of PHSRN–mediated

wound healing enhancement in *db/db* mice (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545).

Example 5

Histological analysis of wound reepithelialization and provisional matrix formation

On day 0, 27 aged *db/db* mice or *db/+* mice are wounded in duplicate with a 4 mm biopsy punch as described above. Mice are divided into 3 treatment groups of 8 mice. The wounds of each group are treated once with 5 µl NS containing either 10 µ g Ac-PRCGVPDVAQF-NH_2 (SEQ ID NO:1) (from MMP–1), Ac-PRSGVPDVAQF-NH_2 (SEQ ID NO:7) (the corresponding C–to–S mutant sequence, or NS. On days 1 through 9, a mouse from each treatment group is sacrificed. Its wounds are dissected out, each surrounded by unwounded skin. Thus, all the wounded mice are assayed in this experiment. Wounds are fixed for at least 3 days in 5% formaldehyde in PBS, and embedded in paraffin.

Please insert the attached Sequence Listing into the specification after the abstract.